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13. ABSTRACT ***(Maximum 200 Words)***

Recent studies indicate that doxorubicin alkylates DNA through catalytic production of formaldehyde and utilization of formaldehyde for attaching itself to G-bases. This discovery prompted the synthesis of anthracycline-formaldehyde conjugates as improved anti-tumor drugs. The first conjugate, Doxoform, was equally toxic to both sensitive and resistant breast cancer cells but is predicted to be hydrolytically too unstable. The second conjugate, Epidoxoform, from reaction of epidoxorubicin with formaldehyde was synthesized and characterized and proved to be less toxic to breast cancer cells but more stable with respect to hydrolysis to epidoxorubicin. The following additional results with Doxoform and Epidoxoform were obtained during the first budget period: 1) The structure of epidoxorubicin-alkylated DNA, solved by x-ray crystallography, shows the epidoxorubicin virtually crosslinking the DNA at NGC sites. 2) Flow cytometry measurements show drug-formaldehyde conjugates are taken up better by both sensitive and resistant breast cancer cells and retained longer than their clinical counterparts. 3) The nucleus of both sensitive and resistant cancer cells is the primary target for drug-formaldehyde conjugates. 4) Drug-formaldehyde conjugates are more toxic to breast cancer cells than confluent mammary epithelial cells.

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Ted H. Kd 9/15/99

PI - Signature Date

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(5) INTRODUCTION

Preliminary extra cellular experiments indicated that the anthracycline antitumor drug, doxorubicin, covalently bonds to DNA through its catalysis of formaldehyde production. Subsequently, it utilizes formaldehyde for covalent attachment to DNA from its 3'-amino group to the 2-amino group of a G-base. This mechanistic understanding prompted the synthesis of anthracycline formaldehyde conjugates as improved antitumor drugs. The first conjugate synthesized, Doxoform, proved to be highly toxic to sensitive and resistant breast cancer cells; however, Doxoform is rapidly hydrolyzed to doxorubicin under physiological conditions. The purpose and scope of the research are to design and synthesize a hydrolytically more stable anthracycline-formaldehyde conjugate, to establish that anthracyclines derive at least some of their toxicity to tumor cells from covalent bonding to DNA, and to determine why anthracycline-formaldehyde conjugates overcome at least some resistance mechanisms.

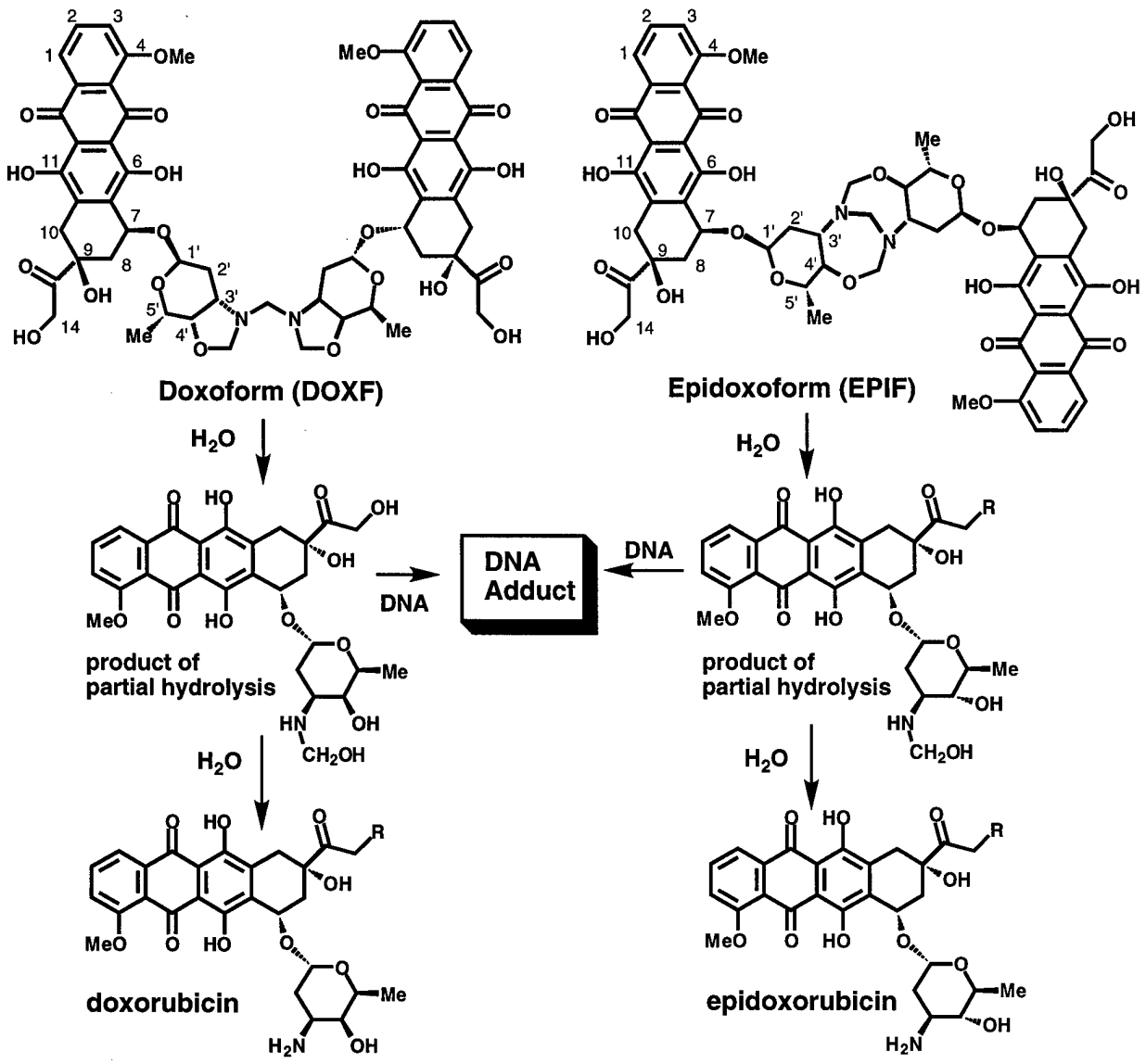
(6) BODY

Research Accomplishments

The following questions raised in the proposal have been addressed during the current budget period. The order is presented differently than in the proposal because of the accomplishments. In particular, a more stable anthracycline-formaldehyde conjugate was discovered early on and hence became the focus of attention.

Can a more stable doxorubicin-formaldehyde conjugate be designed and synthesized?

Doxoform has a predicted half-life of less than 10 min in the vascular system with respect to hydrolysis to doxorubicin and formaldehyde. This half-life was predicted to be too short for eventual use of Doxoform in humans. As a result design and synthesis of alternate formaldehyde conjugates are being explored. The epimer of doxorubicin, epidoxorubicin, was reacted with formaldehyde to yield a dimeric conjugate, Epidoxoform, with a diazadioxabicyclic structure. This structure contrasts with that of Doxoform which is a dimeric conjugate with bisoxazolidinylmethane structure. The structural difference results from the stereochemistry at the 4'-position. Epidoxoform has a predicted half-life of more than 2 h in the vascular system with respect to hydrolysis to epidoxorubicin and formaldehyde. The IC50 values (concentrations which inhibit half the growth) for 3 h Epidoxoform treatment of MCF-7 and MCF-7/Adr cells are 65 and 70 nmolar equiv/L relative to 200 and >10,000 for epidoxorubicin. Furthermore, preincubation of Epidoxoform in cell culture medium containing 10% fetal bovine serum for 6 h at 37 °C increased the IC50 value for MCF-7/ADR treated cells only to 300 nmolar equiv/L. Although the IC50 value for Doxoform is approximately 50-fold lower, Doxoform loses all of its activity against MCF-7/ADR cells in less than 30 min of preincubation.



Do Doxoform and Epidoxoform actually form a covalent bond to DNA in sensitive and resistant breast cancer cells and how do they circumvent resistance?

Because of the emerging importance of Epidoxoform, the structure of the covalent adduct from reaction of epidoxorubicn and formaldehyde with DNA was determined by crystallography. The adduct from reaction of epidoxorubicin and formaldehyde with the self complementary deoxyoligonucleotide CGCGCG was crystallized and the structure solved by molecular replacement. Comparison with the x-ray structure of the adduct from reaction of daunorubicin and formaldehyde with the same deoxyoligonucleotide showed an additional hydrogen bonding interaction from the epimeric 4'-hydroxyl group to the bonded DNA strand; however, hydrogen bonding interactions to the opposing strand appeared to be weaker.

Indirect evidence for formation of covalent bonds to DNA in cells was obtained from a tritium labeling experiment. Doxoform was synthesized from tritiated formaldehyde. Sensitive (MCF-7) and resistant (MCF-7/ADR) cells were treated with tritiated Doxoform for 1 h. Treatment with doxorubicin + tritiated formaldehyde or tritiated formaldehyde for 1 h were used as controls. Cells were lysed and the contents separated into DNA, RNA, and protein fractions, and the fractions were counted in a scintillation counter. Both sensitive and resistant cells treated with tritiated Doxoform showed approximately twice as many counts in DNA than cells treated with doxorubicin + tritiated formaldehyde or with tritiated formaldehyde. Hence, tritiated formaldehyde appears to reach the nucleus of both sensitive and resistant breast cancer cells.

Uptake and release of drug from doxorubicin, Doxoform, epidoxorubicin, and Epidoxoform treated MCF-7 and MCF-7/ADR cells was measured by flow cytometry using drug fluorescence as a measure of drug in cells. MCF-7 cells took up more of all four drugs than MCF-7/ADR cells, and both cell lines took up substantially more of the formaldehyde conjugates than the clinical drugs. Furthermore, both cell lines retained the conjugates hours after drug treatment while the clinical drugs were expelled within 1 h of drug treatment. The rate of uptake of formaldehyde conjugates appeared to parallel the rate of partial hydrolysis to monomeric forms. The half-life for this partial hydrolysis of Epidoxoform is 2 h and for Doxoform less than 10 min.

The location of drug in cells was studied by fluorescence microscopy. The nucleus was the primary location of drug fluorescence from treatment of MCF-7 cells with doxorubicin, Doxoform, epidoxorubicin, or Epidoxoform. Similar strong fluorescence was observed in the nuclei of MCF-7/ADR cells treated with Doxoform or Epidoxoform; however, drug fluorescence was very weak in MCF-7/ADR cells treated with doxorubicin or epidoxorubicin. The rate of appearance of drug fluorescence in cells treated with the formaldehyde conjugates paralleled the rate of partial hydrolysis which paralleled the rate of drug uptake observed by flow cytometry.

The conclusions from the structural determination, tritium labeling experiment, flow cytometry measurements, and fluorescence microscopy are that drug-formaldehyde conjugates are more toxic to both sensitive and resistant breast cancer cells because more of the conjugates reach the nucleus and are retained longer. Further, dimeric conjugates must partially hydrolyze to monomeric conjugates before drug uptake occurs. A structural difference which may permit higher levels of drug uptake is the lack of positive charge on the conjugates in their monomeric form. Conjugation with formaldehyde is predicted to change the pKa such that the drugs are not protonated at physiological pH.

How toxic are Doxoform and Epidoxoform to non-malignant cells?

Doses of Doxoform and doxrubicin which killed 50% of breast cancer cells (MCF-7) and human mammary epithelial cells (HME) (LC50) were established. The measurement with HME cells was performed at confluence and with MCF-7 cells, at non confluence to reflect their natural states. The LC50 for HME cells treated with doxorubicin was 12-fold greater than for MCF-7 cells; correspondingly, the LC50 for HME cells treated with Doxoform was 6-fold greater than for MCF-7 cells. Similar results were observed in a comparison of Epidoxoform with

epidoxorubicin. Hence, these cell experiments showed similar selectivity for the anthracycline and its formaldehyde conjugate in killing breast cancer cells.

Training Accomplishments

Funds have been used for the support of three graduate students performing thesis research toward advanced degrees, two in organic chemistry and one in pharmaceutical sciences.

(7) APPENDICES

1) key research accomplishments

Epidoxoform, the dimeric formaldehyde conjugate of epidoxorubicin, is hydrolytically more stable with respect to complete hydrolysis to the clinical drug than Doxoform, the dimeric formaldehyde conjugate of doxorubicin.

Anthracycline-formaldehyde conjugates are equally toxic to sensitive and resistant breast cancer cells.

Anthracycline-formaldehyde conjugates are taken up better and retained longer by sensitive and resistant breast cancer cells than their clinical counterparts.

The nucleus is the primary target of anthracycline-formaldehyde conjugates in sensitive and resistant breast cancer cells.

The formaldehyde of Doxoform reaches DNA in cells.

Dimeric anthracycline-formaldehyde conjugates must partially hydrolyze to monomeric anthracycline-formaldehyde conjugates before they are taken up by breast cancer cells.

2) reportable outcomes

- manuscripts

Taatjes, D. J.; Fenick, D. J.; Koch, T. H, "Nuclear Targeting and Nuclear Retention of Anthracycline-Formaldehyde Conjugates Implicates DNA Covalent Bonding in the Cytotoxic Mechanism of Anthracyclines" *Chem Res Toxicol..*, *12*, 588-596 (1999). This paper was featured on the cover of the July 1999 issue of the journal.

Podell, E. R.; Harrington, D. J.; Taatjes, D. J.; Koch, T. H. "Crystal Structure of Epidoxoform-formaldehyde *Virtual Crosslink* of DNA and Evidence for Its Formation in Human Breast Cancer Cells" *Acta Cryst. D*, in press (1999).

- abstracts

"Antracycline-Formaldehyde Conjugates: Growth Inhibition, Nuclear Uptake, and Retention in Breast and Prostate Cancer Cells", T. H. Koch, D. J. Taatjes, D. J. Fenick, American Chemical Society National Meeting, Anaheim, CA, March 1999.

- presentations

"Antracycline-Formaldehyde Conjugates: Growth Inhibition, Nuclear Uptake, and Retention in Breast and Prostate Cancer Cells", T. H. Koch, D. J. Taatjes, D. J. Fenick, American Chemical Society National Meeting Symposium: "ANTHRACYCLINE ANTIBIOTICS: FROM THE BENCH TO THE CLINIC" Anaheim, CA, March 1999.

American Chemical Society
Division of Carbohydrate Chemistry

ABSTRACTS

217TH ACS NATIONAL MEETING
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MARCH 21-25, 1999

C. F. Brewer, Program Chair
Z. J. Witczak, Program Secretary

030.

THIRTY YEARS AFTER DOXORUBICIN. GLYCOSIDE ANALOGS AND NEW DERIVATIVES OF ANTHRACYCLINES. Federico Arcamone, Menarini Ricerche, Pomezia,(Rome) I-00040 and ICOCEA-CNR, Bologna, I-40129, Italy

Biosynthetic anthracyclines represent an important example of molecular diversity of natural substances. The development of daunorubicin and doxorubicin and of their synthetic analogs epirubicin and idarubicin has provided pharmacological agents now of established clinical use for the medical treatment of human cancer. More recently, novel disaccharide analogs have been synthetised and one compound, MEN 10755, is currently in clinical trials. Lastly, new anthracycline conjugates as potential anti-gene agents have been obtained and studied.

031.

ANTHRACYCLINE-FORMALDEHYDE CONJUGATES: GROWTH INHIBITION, NUCLEAR UPTAKE, AND RETENTION IN BREAST AND PROSTATE CANCER CELLS. **T. H. Koch,** D. J. Taatjes, and D. J. Fenick, Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309-0215

The clinical anthracycline antitumor drugs, doxorubicin, daunorubicin and epidoxorubicin, catalyze production of formaldehyde which mediates drug-covalent bonding to DNA. Synthetic drug-formaldehyde conjugates show enhanced toxicity to both sensitive and resistant breast and prostate cancer cells relative to the clinical drugs. Fluorescence microscopy shows the nucleus to be the primary target, and experiments with conjugate prepared from tritiated formaldehyde establishes that the formaldehyde bonds to cellular DNA. Flow cytometry shows enhanced uptake and retention of the conjugates relative to the clinical drugs with the effect most dramatic in the more resistant tumor cells. The results implicate drug-DNA adducts in tumor cell growth inhibition and suggest that conjugates are prodrugs to the active metabolites of the clinical drugs.

032.

DESIGN AND EVALUATION OF NOVEL ANTHRACYCLINE-BASED DRUGS. W. Priebe, I. Fokt, T. Przewloka, M. Krawczyk, G. Grynkiewicz, J.B. Chaires, and R. Perez-Soler, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030.

We have prepared three different classes of anthracycline-based drugs designed to (1) overcome clinically relevant multidrug resistance (MDR1- and MRP-related), (2) bind extremely tightly to extended DNA sequences (bisintercalation), and (3) alkylate DNA in a base-specific and regioselective manner. Together, these three classes of drugs represent a unique, mechanistically differentiated set of anticancer agents. Biological evaluations indicated that a new generation of drugs able to circumvent P-gp- and MRP-mediated efflux was significantly more active than doxorubicin (DOX) against MDR tumors, whereas compounds able to alkylate DNA were also dramatically more potent, from 50- to more than 120,000-fold more cytotoxic than DOX. On the other hand, analogs that could tightly bind DNA appeared to be more selective than DOX. Supported in part by NCI grant # CA50270 and Texas Higher Education Board grant ATP00015-090.

033.

BCL-2 EXPRESSION FAILS TO BLOCK APOPTOSIS INDUCED BY THE PROTEIN KINASE C (PKC) INHIBITOR N-BENZYLADRIAMYCIN-14-VALERATE (AD 198 L. Lothstein, C.M. Herring, J.B. Roaten, T.W. Sweatman, J.L. Cleveland*, M. Israel, and M.S. Steiner. Univ. of Tennessee College of Medicine, Memphis, TN 38163; and *St. Jude Children's Research Hospital., Memphis, TN 38105

The anti-apoptotic activity of Bcl-2 is modulated at least in part by ser/thr phosphorylation at multiple sites. One model of regulation suggests that PKC-mediated phosphorylation at ser-70 activates Bcl-2, but can also lead to inactivation by hyperphosphorylation. The doxorubicin (DOX) congener, AD 198, is a potent PKC inhibitor that can competitively inhibit phorbol ester binding to PKC. AD 198 cytotoxicity was compared

Nuclear Targeting and Nuclear Retention of Anthracycline–Formaldehyde Conjugates Implicates DNA Covalent Bonding in the Cytotoxic Mechanism of Anthracyclines

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Nuclear Targeting and Nuclear Retention of Anthracycline−Formaldehyde Conjugates Implicates DNA Covalent Bonding in the Cytotoxic Mechanism of Anthracyclines

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Received January 15, 1999

The anthracycline, antitumor drugs doxorubicin (DOX), daunorubicin (DAU), and epidoxorubicin (EPI) catalyze production of formaldehyde through induction of oxidative stress. The formaldehyde then mediates covalent bonding of the drugs to DNA. Synthetic formaldehyde conjugates of DOX, DAU, and EPI, denoted Doxoform (DOXF), Daunoform (DAUF), and Epidoxoform (EPIF), exhibit enhanced toxicity to anthracycline-sensitive and -resistant tumor cells. Uptake and retention of parent anthracycline antitumor drugs (DOX, DAU, and EPI) relative to those of their formaldehyde conjugates (DOXF, DAUF, and EPIF) were assessed by flow cytometry in both drug-sensitive MCF-7 cells and drug-resistant MCF-7/ADR cells. The MCF-7 cells took up more than twice as much drug as the MCF-7/ADR cells, and both cell lines took up substantially more of the formaldehyde conjugates than the parent drugs. Both MCF-7 and MCF-7/ADR cells retained fluorophore from DOXF, DAUF, and EPIF hours after drug removal, while both cell lines almost completely expelled DOX, DAU, and EPI within 1 h. Longer treatment with DOX, DAU, and EPI resulted in modest drug retention in MCF-7 cells following drug removal but poor retention of DOX, DAU, and EPI in MCF-7/ADR cells. Fluorescence microscopy showed that the formaldehyde conjugates targeted the nuclei of both sensitive and resistant cells, and remained in the nucleus hours after drug removal. Experiments in which [^{3}H]Doxoform was used, synthesized from doxorubicin and [^{3}H]-formaldehyde, also indicated that Doxoform targeted the nucleus. Elevated levels of ^{3}H were observed in DNA isolated from [^{3}H]Doxoform-treated MCF-7 and MCF-7/ADR cells relative to controls. The results implicate drug−DNA covalent bonding in the tumor cell toxicity mechanism of these anthracyclines.

Introduction

The anthracycline, antitumor drugs doxorubicin (DOX)[1] and daunorubicin (DAU) remain an important part of chemotherapy regimens in the clinic. Epidoxorubicin (EPI), the 4′-epimer of doxorubicin, is also a widely used chemotherapeutic agent that is marketed worldwide except in the United States (*1*). DNA is an important target for the anthracyclines, with induction of topoisomerase II-mediated strand breaks as a cytotoxic consequence (*2*, *3*). The drugs are high-affinity DNA intercalators (*4*) and are known to form unstable covalent bonds to extracellular DNA when activated under redox conditions (*5*, *6*). Two types of covalent bonding based upon differences in stability have been described: more stable drug−DNA cross-links and less stable drug−DNA adducts (*7*). With previous extracellular experiments, we established that DOX, DAU, and EPI promote the production of formaldehyde through induction of oxidative stress in the presence of ferric ion (*8*, *9*). The iron-chelating ability of the anthracyclines is proposed to be an important factor for the efficient production of formaldehyde near the drug for formation of a drug−formaldehyde conjugate at the drug's 3′-amino substituent. This intermediate is thought to be the active drug metabolite which bonds to DNA forming a covalent methylene linkage to the 2-amino group of a G-base as shown in Scheme 1. At 5′-NGC-3′ sites, drug intercalation, covalent bonding, and hydrogen bonding at the C9 hydroxyl combine to form a *virtual DNA cross-link* (Scheme 1) (*8*, *10*−*12*). This *virtual DNA cross-link* corresponds to the drug−DNA cross-link described earlier (5−7). The drug−DNA adduct is presumed to have a similar structure without the hydrogen bonding interaction from the C9 hydroxyl to the G-base on the opposing strand. Throughout this paper, the term drug−DNA adduct is used to describe both types of drug−DNA bonding. Covalent attachment of DOX, DAU, or EPI to DNA is likely to be at least partially responsible for the drugs' toxicity to tumor cells (*13*−*15*), possibly in conjuction with its effect on the topoisomerase−DNA complex.

A major problem associated with cancer chemotherapy with the anthracyclines as well as other antitumor drugs is specific resistance and multidrug resistance. Multiple

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[1] Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; DAU, daunorubicin; DAUF, Daunoform; DMSO, dimethyl sulfoxide; DOX, doxorubicin; DOXF, Doxoform; EPI, epidoxorubicin; EPIF, Epidoxoform; FBS, fetal bovine serum; PBS, 0.1 M phosphate-buffered saline (pH 7.4); Pgp, P-170 glycoprotein; SDS, sodium dodecyl sulfate; WGA-FITC, wheat germ agglutinin fluorescein-linked isothiocyanate.

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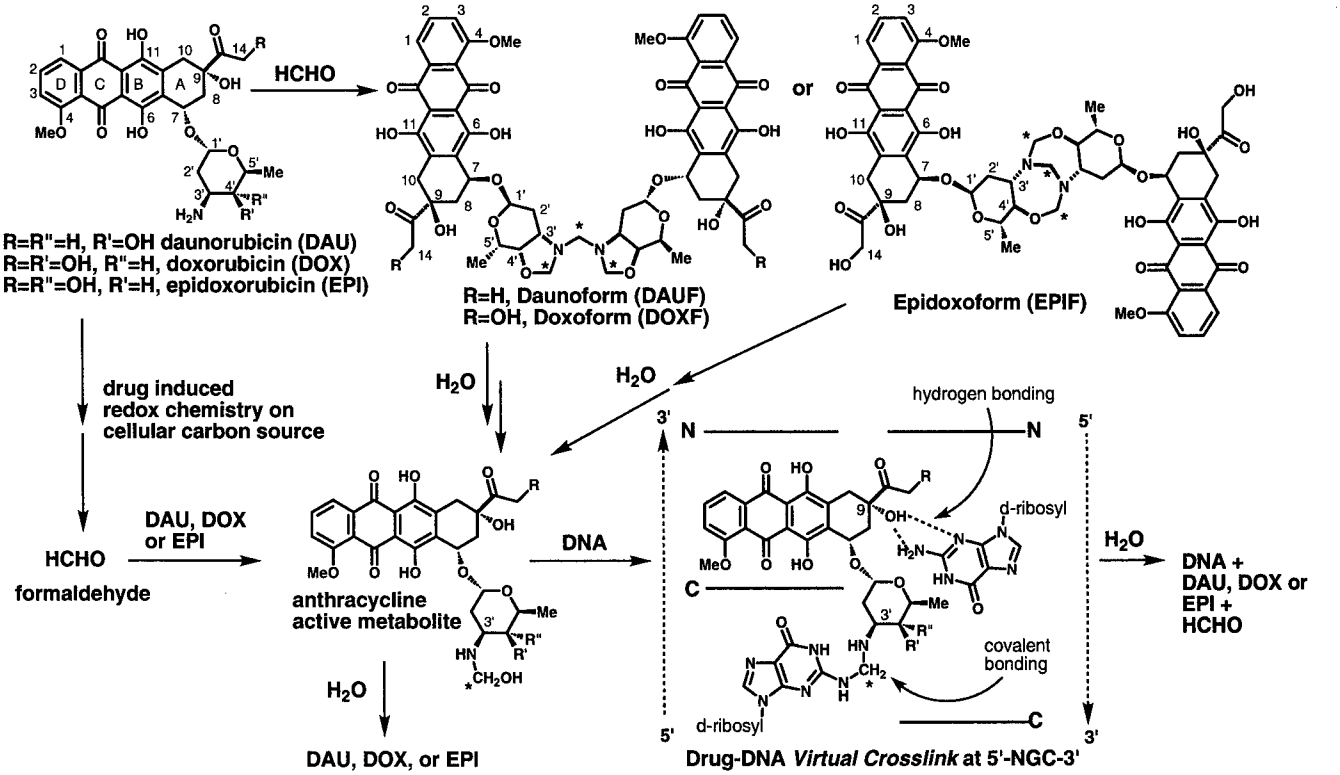
Scheme 1. Structures of Parent Drugs (DAU, DOX, and EPI), Their Conversion to Formaldehyde Conjugates (DAUF, DOXF, and EPIF), Formation of Anthracycline Active Metabolites, and Formation and Hydrolysis of a Drug–DNA Virtual Cross-Link[a]



[a] Carbons coming from formaldehyde are denoted with asterisks.

resistance mechanisms have been discovered in cancer cells, with the best characterized being the overexpression of the drug efflux pump P-170 glycoprotein (Pgp) (*16*, *17*). Another resistance mechanism relevant to the discussion here is the altered activity of redox enzymes involved in anthracycline-associated oxidative stress. Enzymes which produce reactive oxygen species (e.g., cytochrome P450 reductase) exhibit reduced activity, and enzymes which neutralize reactive oxygen species (e.g., superoxide dismutase and gluthathione peroxidase) exhibit increased activity (*18–20*). Both of these mechanisms are active in MCF-7/ADR cells (*18*, *21*), the resistant human breast cancer cells employed in the studies described here.

Realizing the probable importance of formaldehyde in the tumor cell toxicity of DOX, DAU, and EPI, we synthesized formaldehyde conjugates of the drugs, denoted Doxoform (DOXF), Daunoform (DAUF), and Epidoxoform (EPIF) (*11*, *22*). These conjugates are prodrugs to the anthracycline active metabolites, as shown in Scheme 1. Cytotoxicity experiments revealed that DOXF, DAUF, and EPIF were significantly more toxic to both sensitive (MCF-7) and resistant (MCF-7/ADR) breast cancer cells than the parent compounds DOX, DAU, and EPI (*11*, *22*). Here we report the uptake, retention, and distribution of DOX, DAU, and EPI with respect to their formaldehyde conjugates in MCF-7 and MCF-7/ADR cells. Drug uptake and retention were assessed by flow cytometry, and the distribution of the drugs was visualized by fluorescence microscopy. Drug distribution was also studied by treating cells with ^{3}H-labeled DOXF and analyzing isolated DNA, RNA, and protein by scintillation counting. The results implicate DNA alkylation in the cell-killing mechanism of these drugs.

Experimental Procedures

Materials. All tissue culture materials were obtained from Gibco Life Technologies (Grand Island, NY) unless otherwise stated. MCF-7 breast cancer cells were obtained from American Type Culture Collection (Rockville, MD), and MCF-7/ADR adriamycin-resistant breast cancer cells were a gift from W. W. Wells (Michigan State University, East Lansing, MI). Daunoform (DAUF), Doxoform (DOXF), and Epidoxoform (EPIF) were synthesized from daunorubicin, doxorubicin, and epidoxorubicin, respectively, by reaction with formaldehyde as described previously (*11*, *22*). These drug–formaldehyde conjugates are toxic materials and should be handled with caution especially when dissolved in DMSO which potentially makes them more permeable to the skin. Concentrations of drug–formaldehyde conjugates are given in micromolar equivalents per liter to correct for the conjugates bearing two active drug molecules per structural unit. DAPI, 4,6-diamidino-2-phenylindole, and wheat germ agglutinin fluorescein-linked (WGA–FITC) were from Sigma Chemical Co. (St. Louis, MO). TRI reagent for separation of cellular DNA, RNA, and protein was from Molecular Research Center, Inc. (Cincinnati, OH). Tritiated aqueous formaldehyde was obtained from DuPont-NEN Research Products (Boston, MA).

Maintenance of Cell Lines. MCF-7 and MCF-7/ADR cell lines were maintained in vitro by serial culture in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gemini Bio-Products, Calbasas, CA), L-glutamine (2 mM), HEPES buffer (10 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Assessment of Drug Uptake and Release by Flow Cytometry. Cultured cells (MCF-7 and MCF-7/ADR) were dissociated with trypsin–EDTA and plated in six-well plates (ca. 500 000 cells/well) and allowed to adhere overnight. The cells were treated with 0.5 μmolar equiv/L drug (DOX, DAU, EPI, DOXF, DAUF, and EPIF) at 37 °C for various amounts of

time (5 min, 30 min, 1 h, 2 h, and 3 h). For each time point, the medium was removed and the cells were trypsinized and suspended in 3 mL of RPMI 1640 medium without phenol red and without FBS. The cells were transferred to 15 mL conical vials and centrifuged at 1000 rpm for 5 min at 10 °C. The supernatant was removed and replaced with 1 mL of RPMI 1640 medium without phenol red and without FBS. The cells were kept at 0 °C until analysis (up to 3 h). Control experiments exhibited no significant loss of fluorescence in samples kept at 0 °C for up to 4 h.

For drug retention analysis, cells were plated as described and treated with 0.5 μmolar equiv/L drug for 1 h (or 24 h). EPIF treatment lasted 3 h to allow for sufficient hydrolysis and drug–DNA adduct formation. Following drug treatment, the cells were incubated in fresh, drug-free medium for various time periods (0 min, 5 min, 30 min, 1 h, 3 h, and 6 h). After the alotted time had passed, the cells were prepared for flow cytometry analysis as described above.

The extent of drug uptake was determined by flow cytometry as previously described (*23*). All flow cytometry measurements were made with a Becton Dickinson (Franklin Lakes, NJ) FACScan flow cytometer, using a Hewlett-Packard 9000 series model 340 computer for data storage and analysis. Drug-treated cells were analyzed with excitation at 488 nm (15 mW Ar ion laser), with emission monitored between 570 and 600 nm. Instrument settings were held constant for all experiments, and 5000 cells were counted per measurement. The emission of drug-free cells was similarly measured to determine background fluorescence. The final data were plotted as mean fluorescence (as determined by computer data analysis) versus drug incubation time (or recovery time) for ease of data representation.

Analysis of Intracellular Drug Distribution by Fluorescence Microscopy. Cells were plated in six-well plates (ca. 300 000 cells/well). Each well contained a sterile cover slip, and the cells were allowed to adhere to the cover slip overnight. Each well contained 3 mL of RPMI 1640 medium. The drugs (DOX, DAU, EPI, DOXF, DAUF, and EPIF) were dissolved in DMSO to a concentration of 50 μmolar equiv/L (100-fold concentration). Then, 30 μL of each DMSO solution was added to the appropriate well, resulting in a 100-fold dilution (0.5 μmolar equiv/L drug and 1% DMSO). The cells were incubated with the drug for 5 min, 30 min, 1 h, 2 h, or 3 h. Following drug treatment, the medium was removed and the cells were washed with 2 mL of PBS. The cells were then fixed to the cover slips by submerging in 3 mL of cold (−20 °C) methanol and storing on ice for 5 min. The methanol was removed, and the cells were washed with 3 mL of PBS. The cover slips were then removed from the wells and inverted on a drop (30 μL) of DAPI (0.2 μg/mL in PBS) placed on Parafilm. The cover slips were kept on the DAPI solution for 5 min at ambient temperature. The cover slips were then rinsed with PBS and mounted on a microscope slide using a drop (30 μL) of mowiol mounting medium. The slides were allowed to dry in the dark overnight. Microscopic images were observed at a magnification of 1000× and recorded with a Zeiss Axioplan (Carl Zeiss, Thornwood, NY) fluorescence microscope equipped with a Photometrics Sensys (Tucson, AZ) digital CCD camera system. Images were developed using IP-LAB Spectrum Software. Drug fluorescence was observed at wavelengths above 590 nm with excitation at 546 ± 6 nm, and DAPI fluorescence was observed at wavelengths above 420 nm with excitation at 355 ± 20 nm.

MCF-7 and MCF-7/ADR cells were each treated with fluorescein-linked wheat germ agglutinin to visualize the Golgi apparatus. Fluorescein fluorescence was observed at wavelengths above 515 nm with excitation at 470 ± 20 nm.

For drug retention experiments with short periods of drug exposure, the cells were treated with drugs for 1 h except for EPIF, where cells were treated for 3 h to allow sufficient time for EPIF hydrolysis and drug–DNA adduct formation. For the assessment of retention after longer exposure, cells were treated with drugs for 24 h. After drug treatment, cells were incubated in fresh medium for various time periods (0 min, 5 min, 30 min, 1 h, 3 h, and 6 h). Cells were then fixed, stained with DAPI, and mounted as described above.

[^{3}H]Doxoform Synthesis. To a 6.7 mM solution of doxorubicin (19.4 mg in 5 mL) in pH 6 sodium cacodylate buffer (30 mM) were added 0.05 mmol of [^{3}H]H_2CO (5 mCi) and 1.62 mmol of H_2CO (3% [^{3}H]H_2CO). The mixture was stirred at room temperature for 20 min, and then extracted with 2 × 30 mL of chloroform. The chloroform extracts were dried over sodium sulfate and combined. The solvent was then removed by rotary evaporation. [^{3}H]Doxoform was crystallized by redissolving the solid in 500 μL of chloroform and transferring the mixture to a stoppered vial. To this were added 2 mL of *n*-hexane and 8 mL of ethyl acetate. The vial was stored in the dark at ambient temperature until crystal formation was complete (5 days). The crystals were washed with *n*-hexane and dried. The material was stored at ambient temperature until it was used. Scintillation counting gave 22 502 cpm for 4.46 nmol of the crystalline [^{3}H]Doxoform.

Cell Experiments with [^{3}H]Doxoform and [^{3}H]Formaldehyde. MCF-7 and MCF-7/ADR cells (approximately 10 million cells) in RPMI 1640 medium were treated for 1 h with 1 μmolar equiv/L [^{3}H]Doxoform (3% ^3H_2CO) or 1 μM doxorubicin and 1.5 μM HCHO (3% ^3H_2CO) or 1.5 μM HCHO (3% ^3H_2CO). The Doxoform concentration is reported as 1 μmolar equiv/L because it is a dimeric molecule. Doxoform and doxorubicin were introduced as DMSO solutions. All three mixtures contained 0.5% DMSO (100 μL in 20 mL of medium). Incubations were performed at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. After 1 h, the medium was removed and stored at 4 °C until analysis. Isolation of cellular DNA, RNA, and protein was performed with TRI Reagent. For each flask containing 1 × 10^7 cells, 7 mL of TRI Reagent was added and the solution was allowed to sit at ambient temperature for 5 min. The TRI Reagent/cell lysate mixture was then transferred to a 15 mL centrifuge tube, and 700 μL of 1-bromo-3-chloropropane was added. The mixture was shaken vigorously for 15 s and allowed to stand at ambient temperature for 5 min. The solution was centrifuged at 3000 rpm for 15 min at 4 °C. After centrifugation, the solution contained an aqueous phase, an interphase, and an organic phase. The aqueous phase was removed for later isolation of RNA, and the DNA was precipitated from the interphase and the organic phase by the addition of 2.1 mL of 100% ethanol. The samples were mixed by inversion and centrifuged at 2000 rpm for 5 min at 4 °C. The supernatant was removed from the DNA pellet and stored for subsequent protein isolation. The DNA was washed twice with 7 mL of 0.1 M sodium citrate solution containing 10% ethanol. With each wash, the DNA pellet was stored in the washing solution for 30 min at 0 °C with periodic mixing. The sample was centrifuged at 2000 rpm for 5 min at 4 °C after each wash. Following the sodium citrate washes, a final wash with 7 mL of 75% ethanol was performed, with immediate centrifugation at 2000 rpm for 5 min at 4 °C. The ethanol was removed, and the DNA pellet was dissolved in 400 μL of 8 mM sodium hydroxide. RNA was precipitated from the previously extracted aqueous phase by addition of 3.5 mL of 2-propanol. The sample was mixed by inversion and stored at ambient temperature for 10 min and centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was removed, and the RNA pellet was washed with 70% ethanol followed by centrifugation at 3000 rpm for 5 min at 4 °C. The supernatant was then removed and the RNA pellet suspended in 500 μL of water (autoclaved and 0.2 μm filtered). DNA and RNA were quantitated by measuring the absorbance at 260 nm, assuming 1 OD_{260} = 50 μg of dsDNA/mL. The 260 nm/280 nm absorbance ratio for DNA isolates was >1.6; for RNA, the ratio was >1.9. The concentration of the DNA or RNA solutions for analysis by scintillation counting was ca. 300 μg/mL. Cellular proteins were precipitated from the phenol/ethanol supernatant (from the DNA precipitation) upon the addition of 8 mL of 2-propanol. The samples were stored at ambient temperature for 15 min and centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was removed, and the protein pellet was washed

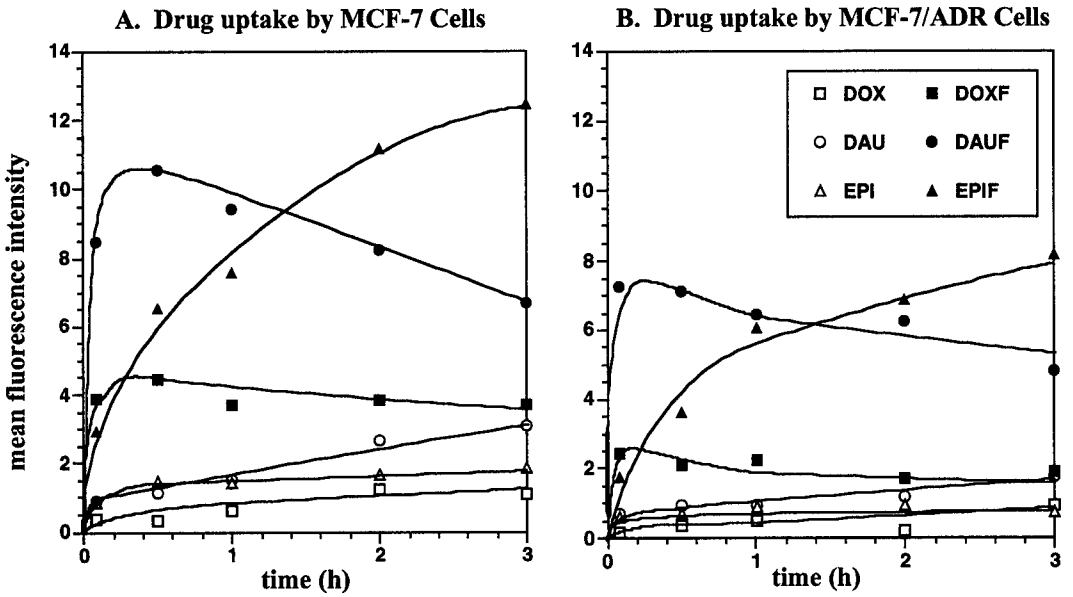


Figure 1. Uptake (A and B) of DOX, DAU, EPI, DOXF, DAUF, and EPIF by sensitive MCF-7 and resistant MCF-7/ADR tumor cells. Drug uptake by cells treated with 0.5 μmol equiv/L drug was observed as a function of time by flow cytometry by monitoring drug fluorescence at 570–600 nm upon excitation at 488 nm. Drug concentrations are given in micromolar equivalents per liter because DOXF, DAUF, and EPIF are dimeric in drug reactive intermediates.

three times with 14 mL of a 0.3 M guanidine hydrochloride solution in 95% ethanol. The samples were stored in the washing solution for 20–30 min at ambient temperature followed by centrifugation at 3000 rpm for 5 min at 4 °C. Then, the protein pellet was vortexed in 2 mL of 100% ethanol and stored at ambient temperature for 20 min. The sample was then centrifuged at 3000 rpm for 5 min at 4 °C. The supernatant was removed, and the proteins were dissolved in 2 mL of a 1% SDS solution. The protein concentration was approximately 6 mg/mL for each sample as determined by absorption at 280 nm relative to bovine serum albumin standard calibration curve. ^{3}H was counted with a Beckman (Fullerton, CA) LS 3801 scintillation counter. To 3 mL of scintillation fluid (Biosafe AQ–Research Products International Corp., Mount Prospect, IL) was added 300 μL of sample. Each sample was counted for 45 min.

Results

Flow Cytometry. The uptake of 0.5 μmolar equiv/L DOX, DAU, and EPI versus the uptake of their respective formaldehyde conjugates, DOXF, DAUF, and EPIF, by human breast cancer cells was assessed by flow cytometry using drug fluorescence as a measure of drug in cells. The concentration of drug is given in micromolar equivalents per liter because DOXF, DAUF, and EPIF are dimeric in active drug. Both doxorubicin-sensitive MCF-7 cells and doxorubicin-resistant MCF-7/ADR cells were studied. Drug uptake was monitored over a 3 h time period, and the results are plotted in Figure 1 (A and B). After 3 h, EPIF was taken up the most by both MCF-7 and MCF-7/ADR cells followed by DAUF, DOXF, DAU, EPI, and DOX. The sensitive cells took up at least twice as much of the drug–formaldehyde conjugate as the respective parent drug. In resistant cells, less of each drug was taken up; however, the ratio of the uptake of drug–formaldehyde conjugate to parent drug was still at least a factor of 2. The initial rate of uptake of DAUF and DOXF was substantially higher than that by EPIF.

Flow cytometry was also used to quantitate the amount of drug retained in MCF-7 and MCF-7/ADR cells as a function of time following drug treatment. For 1 h of drug treatment (3 h for EPIF), the data (Figure 2A,B) show that the parent compounds are released from both sensitive and resistant cells within 1 h of drug removal, while a significant amount of the formaldehyde conjugates remains in the cells even 6 h after drug removal. For 24 h of drug treatment, the data (Figure 2C,D) show that the parent compounds are still not retained in MCF-7/ADR cells, but are modestly retained in MCF-7 cells.

Fluorescence Microscopy. The location of drug in doxorubicin-sensitive and doxorubicin-resistant MCF-7 cells was observed by fluorescence microscopy, again relying on drug fluorescence as a measure of drug concentration. Cells were treated with 0.5 μmolar equiv/L drug for a variety of time periods as described in Experimental Procedures. All six drugs were investigated as a function of treatment time and time of recovery following drug treatment. The drug-treated cells were additionally treated with the nuclear stain 4,6-diamidino-2-phenylindole (DAPI). Fluorescence microscopy revealed that the formaldehyde conjugates targeted the nuclei of both MCF-7 and MCF-7/ADR cells, as shown with DOXF-treated MCF-7 and MCF-7/ADR cells in Figure 3 (rows a and b). The drug fluorescence mapped the fluorescence of DAPI, indicating primarily nuclear localization of DOXF, DAUF, and EPIF. Substantial amounts of DOXF and DAUF were present in the nuclei of both MCF-7 and MCF-7/ADR cells after only a 5 min drug treatment, while nuclear uptake of EPIF was considerably slower (Figure 4 and data not shown). This is due to the difference in the rates of hydrolysis of DOXF, DAUF, and EPIF to the active forms of the drugs. The parent compounds also targeted the nuclei of MCF-7 cells, but were present in smaller amounts and also were minimally present in the cytoplasm (data not shown). Additional experiments using confocal microscopy yielded results similar to those observed by fluorescence microscopy (data not shown). Control experiments with the Golgi stain, fluorescein-linked wheat germ agglutinin (*24*), established that none of the drugs was predominantly in the Golgi apparatus of either MCF-7 or MCF-

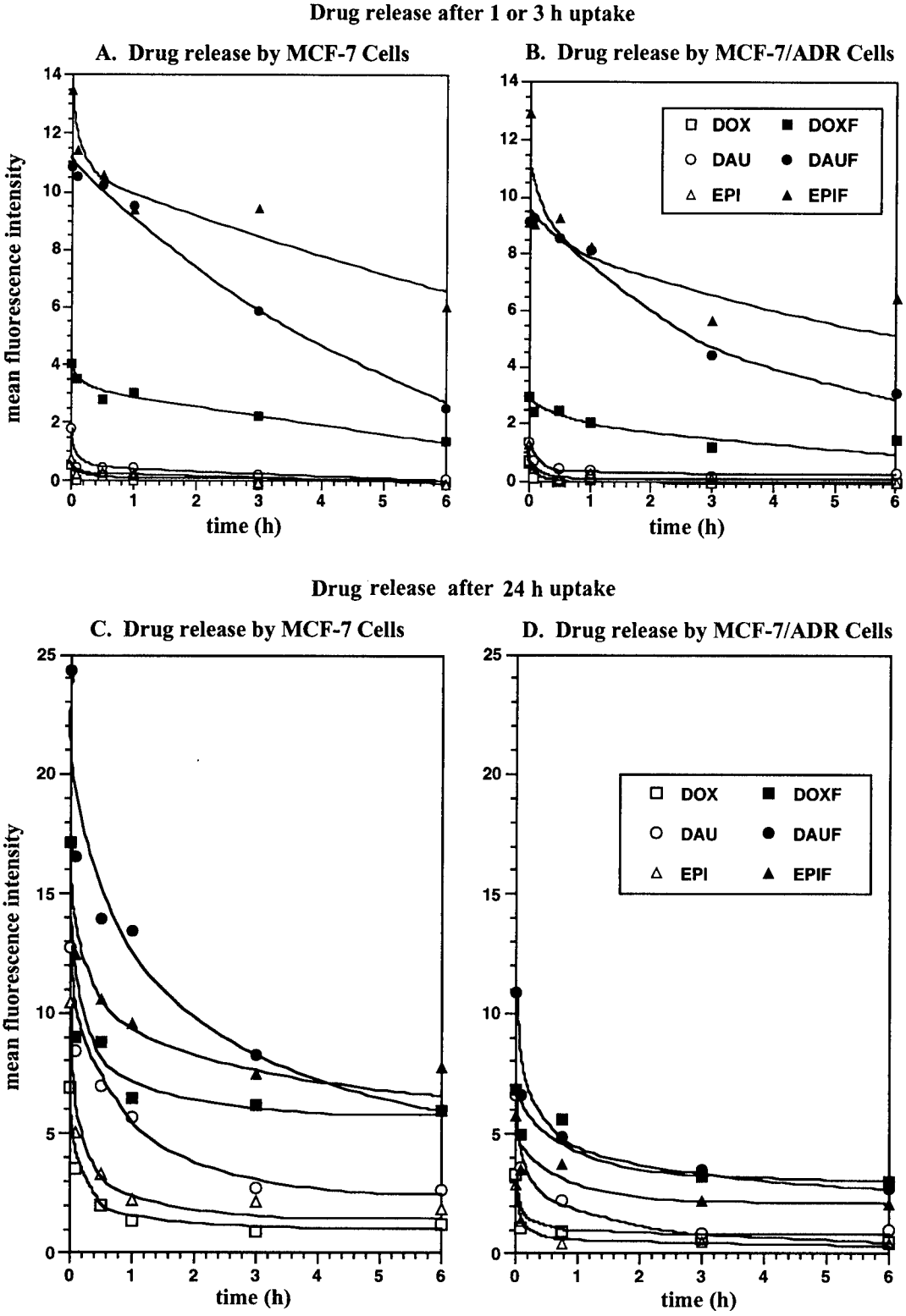


Figure 2. Release of DOX, DAU, EPI, DOXF, DAUF, and EPIF by sensitive MCF-7 (A and C) and resistant MCF-7/ADR (B and D) tumor cells after drug treatment for 1 h (except with EPIF for which drug treatment was 3 h to allow time for its hydrolysis to reactive intermediates) (A and B) and after drug treatment for 24 h (C and D). Drug release was assessed by flow cytometry.

7/ADR cells. Very little of the parent drugs was taken up by MCF-7/ADR cells, and no particular specificity for drug localization was observed (Figure 3, row c).

A major difference between the parent compounds and the formaldehyde conjugates was observed when the cells were incubated in drug-free medium following treatment with drug for 1 h (3 h with EPIF). The formaldehyde conjugates remained in the nuclei of both sensitive and resistant MCF-7 cells, even 6 h after drug removal (Figure 5 and data not shown). The parent drugs, however, were expelled from the cells within 30−60 min of drug removal as shown for DOX-treated cells in Figure 5. Similar results were observed following drug treatment for 24 h (data not shown).

Measurements of Formaldehyde Levels in Cells. Because drug−DNA adducts and drug−formaldehyde

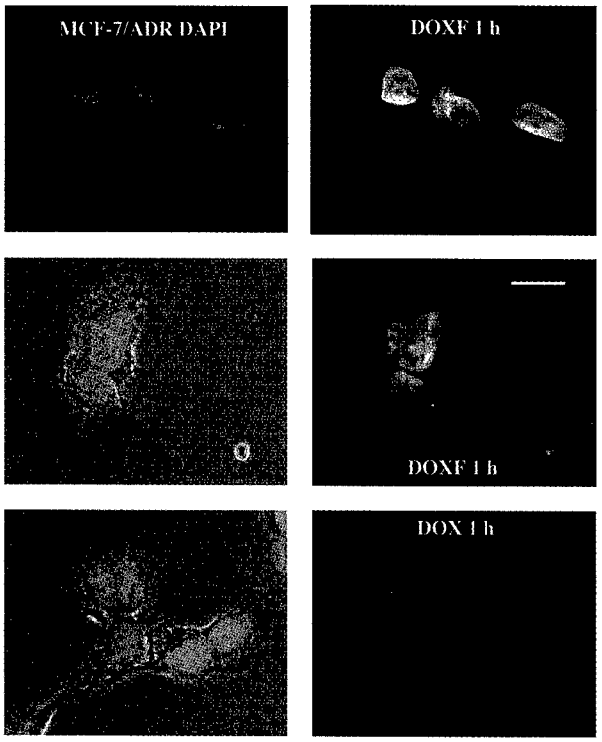


Figure 3. Fluorescence micrographs of resistant MCF-7/ADR tumor cells fixed after exposure to 0.5 μmol equiv/L DOXF (row a) and DOX (row c) for 1 h and sensitive MCF-7 tumor cells fixed after exposure to 0.5 μmol equiv/L DOXF (row b) for 1 h. Both sets of cells were also treated with the nuclear stain DAPI; in rows b and c, DAPI-stained nuclei are superimposed on whole cells. Drug fluorescence was observed at wavelengths above 590 nm with excitation at 546 nm and DAPI fluorescence at wavelengths above 420 nm with excitation at 355 nm; the bar is 25 μm long.

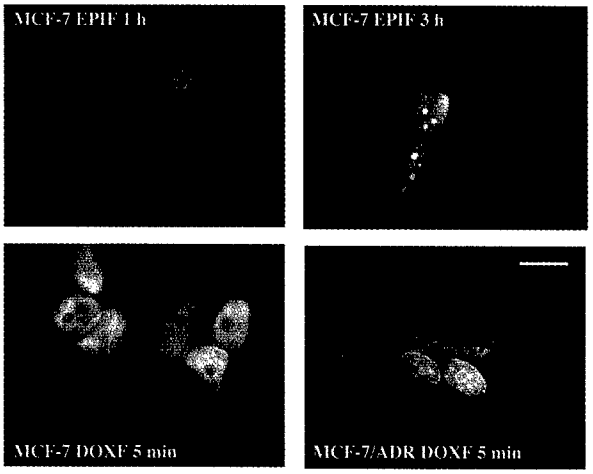


Figure 4. Fluorescence micrographs of sensitive MCF-7 cells treated with 0.5 μmol equiv/L EPIF or DOXF and MCF-7/ADR cells treated with 0.5 μmol equiv/L DOXF, all as a function of time of exposure to drug. Drug was removed from cells at the indicated time points, fixed, and stained with the nuclear stain DAPI. Fluorescence was observed as described in the legend of Figure 3; the bar is 25 μm long.

conjugates are hydrolytically unstable at elevated temperatures (*7*, *25*), most likely with release of formaldehyde, such drug-associated formaldehyde should be detectable. This was achieved using a tritium label at the formaldehyde carbon of DOXF. The distribution of formaldehyde from tritiated DOXF between DNA, RNA, and protein in MCF-7 and MCF-7/ADR cells relative to

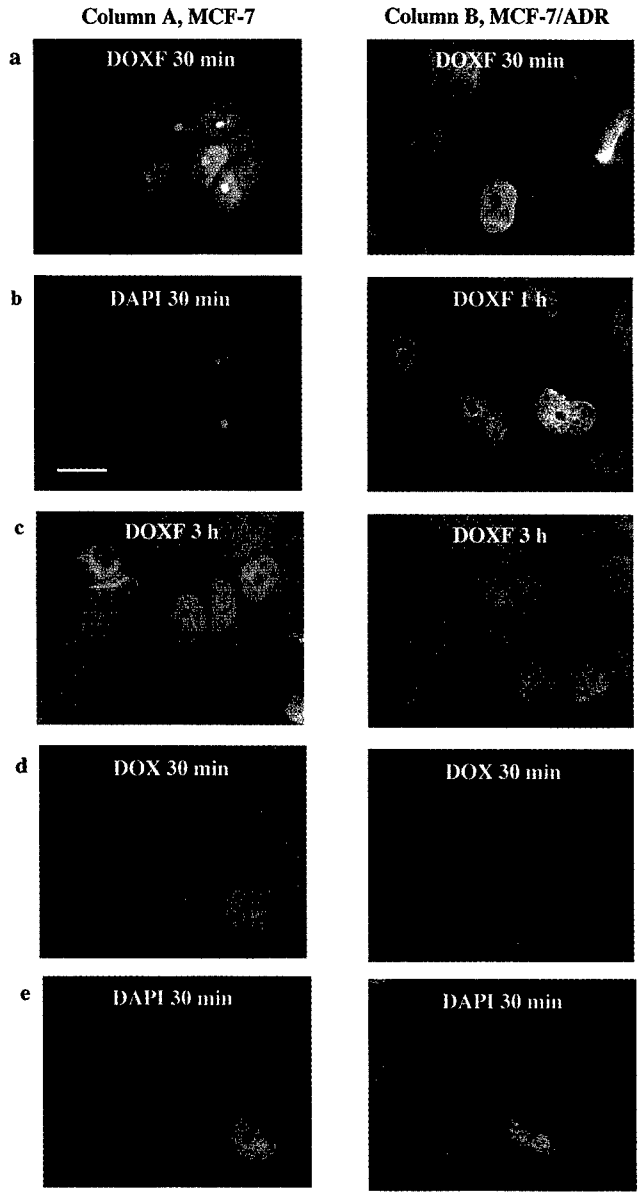


Figure 5. Fluorescence micrographs of MCF-7 cells (column A) and MCF-7/ADR cells (column B) as a function of time for drug release. Cells were treated with either 0.5 μmol equiv/L DOXF or DOX for 1 h and then placed in drug-free medium. At the indicated times in drug-free medium, cells were fixed and stained with DAPI. Fluorescence was observed as described in the legend of Figure 3; the bar is 25 μm long.

controls was determined by treating cells under a variety of conditions, followed by separation of DNA, RNA, and protein and detection by scintillation counting. Cells were treated with 1 μmolar equiv/L [^{3}H]Doxoform (3% ^3H_2CO) or 1 μM doxorubicin and 1.5 μM H_2CO (3% ^3H_2CO) or 1.5 μM H_2CO (3% ^3H_2CO) for 1 h.

Tritiated formaldehyde levels were measured with a scintillation counter for medium, DNA, RNA, and protein. The data, reported in Table 1, show the calculated counts per minute for the total amount of isolated material. In each case, the counts per minute value reported is the counts per minute value above background, which was determined to be 57.6. The counts per minute value for the total amount of isolated material was calculated by dividing the counts per minute value by the fraction of material actually used for scintillation counting. The volume of sample added to each scintillation vial was 300

Table 1. Tritium Label in Units of Counts per Minute per Milligram in DNA, RNA, and Protein of MCF-7 and MCF-7/ADR Cells Treated with either Tritiated Doxoform or Doxorubicin plus Tritiated Formaldehyde or Tritiated Formaldehyde[a]

location of label	MCF/7 with [^{3}H]DOXF	MCF-7 with DOX and ^3H_2CO	MCF-7 with ^3H_2CO	MCF-7/ADR with [^{3}H]DOXF	MCF-7/ADR with DOX and ^3H_2CO	MCF-7/ADR with ^3H_2CO
DNA	725 ± 18 (71)[b]	362 ± 10	376 ± 12	939 ± 19 (41)[b]	279 ± 10	355 ± 11
RNA	31 ± 31 (6)[b]	194 ± 12	214 ± 14	31 ± 39 (21)[b]	169 ± 11	185 ± 14
protein	41 ± 2 (35)[b]	17 ± 1	12 ± 1	23 ± 2 (13)[b]	11 ± 1	10 ± 1

[a] The data are normalized to counts in recovered cell culture media. The errors represent two standard deviations in scintillation counting. [b] The number in parentheses is one standard deviation with respect to reproducibility of the experimental measurement.

μL. This represented 1.5% of the total medium (20 mL), 75% of the DNA (400 μL), 60% of the RNA (500 μL), and 15% of the protein (2 mL). Thus, if the DNA counts per minute value were 73, the counts per minute value for the total isolated material would be 97 (73/0.75). The data in Table 1 show that the distribution of formaldehyde is quite different in [^{3}H]DOXF-treated cells. Substantially more formaldehyde was observed in the DNA of Doxoform-treated cells than in cells treated with doxorubicin and formaldehyde or formaldehyde alone.

Discussion

Covalent Bonding to Nuclear DNA. The results of fluorescence microscopy and tritium labeling experiments indicate that the chromosomal DNA of both sensitive and resistant tumor cells is the primary target for the anthracycline–formaldehyde conjugates, DOXF, DAUF, and EPIF. In particular, Figure 3 (rows a and b) shows that the drug fluorescence mostly overlaps the fluorescence of DAPI, indicating the specific nuclear localization of the fluorophore of DOXF. Only slight drug fluorescence is observed outside the nucleus, and since drug fluorescence is enhanced in lipid membranes (*26*) and partially quenched by drug–DNA intercalation (*27*, *28*), the observation of fluorescence predominantly in the nucleus more certainly identifies it as the key target. Similar results were observed with DAUF and EPIF in both MCF-7 and MCF-7/ADR cell lines (data not shown). Furthermore, elevated levels of ^{3}H were observed in the DNA of [^{3}H]DOXF-treated MCF-7 and MCF-7/ADR cells with respect to controls (Table 1). This concurs with the fluorescence microscopy data and indicates that DOXF (and by analogy DAUF and EPIF) targets the nuclei of cells. Fluorescence microscopy shows that the parent compounds DOX, DAU, and EPI also target the nuclei in MCF-7 cells. However, MCF-7/ADR cells take up very little DOX, DAU, and EPI, with drug mainly in the cytoplasm and very little in the nucleus (Figure 3, row c, and data not shown). Our observations concur with previous reports showing reduced levels of uptake and nuclear exclusion of the drugs in MCF-7/ADR cells (*29*–*31*).

Other differences among the six drugs reside in the rate and quantity of drug uptake and drug release. These differences are apparent from the flow cytometry and fluorescence microscopy studies. EPIF is taken up more slowly than DAUF or DOXF (Figure 1). This slower uptake parallels the observed differences in the rates of hydrolysis of DAUF and DOXF versus EPIF to the active metabolites predicted to be reactive with DNA (Scheme 1). At 37 °C, the half-lives of DAUF and DOXF with respect to formation of the active metabolites at pH 7.4 are less than 10 min (*22*), while the half-life of EPIF at 37 °C with respect to formation of the active metabolite is 2 h (*11*). Flow cytometry shows that the fluorophores of DAUF and DOXF reach the maximum levels in both sensitive and resistant cells in 15 min, while the fluorophore of EPIF continues to rise in both cell lines over a 3 h period (Figure 1A,B). Fluorescence microscopy (Figure 4) qualitatively shows the same result and indicates that the drug is predominantly in the nucleus. Hence, both flow cytometry and fluorescence microscopy indicate that hydrolysis of EPIF to the active metabolite precedes drug uptake and binding to DNA.

The observation that nuclear localization of the fluorophore of DOXF, DAUF, and EPIF is directly related to the rates of hydrolysis to the active metabolites indicates that these formaldehyde conjugates alkylate the chromosomal DNA of MCF-7 and MCF-7/ADR cells. Additional evidence for drug–DNA alkylation by DOXF, DAUF, and EPIF is provided by the drug retention analysis (Figure 2). DOXF-, DAUF-, and EPIF-treated cells placed in fresh, drug-free medium retained drug in the nucleus for hours after treatment. The observed decrease in nuclear drug levels with time is due to hydrolysis of the drug–DNA adducts. Both sensitive and resistant cells exhibited similar levels of retention, consistent with identical IC_{50} values for DOXF, DAUF, and EPIF versus sensitive and resistant cells (*11*, *22*). The parent drugs (DOX, DAU, and EPI), however, were rapidly expelled from the nucleus following treatment for 1 h in both sensitive and resistant cells, indicative of a more labile intercalative interaction with DNA. Extracellular evidence for increased stability of DNA *virtually cross-linked* by doxorubicin versus DNA intercalated by doxorubicin comes from measurements of rates of DNA exchange. DNA strand exchange was inhibited 3.9-fold by a doxorubicin intercalated in a double-stranded oligonucleotide but 637-fold by a doxorubicin *virtually cross-linked* to the oligonucleotide (*12*). When cells were treated with parent drug for 24 h, small amounts of DOX, DAU, and EPI were retained in the nuclei of MCF-7 cells (Figure 2C). This suggests the presence of drug–DNA adducts similar to those formed by DOXF, DAUF, and EPIF. The longer drug treatment (24 h vs 1 h) allows the parent drugs time to generate formaldehyde catalytically (Scheme 1) (*8*, *9*). The formaldehyde then mediates formation of the drug–DNA adducts. Retention of DOX, DAU, and EPI was not seen in resistant cells following drug treatment for 24 h (Figure 2D) due to reduced levels of drug uptake and changes in the activity of enzymes which influence formaldehyde production by these cells (*9*, *18*, *32*). Some of the DOX, DAU, and EPI retained by the MCF-7 cells after treatment for 24 h may also have resulted from drug trapped in TopoII cleavable complexes and/or transcription complexes. Formation of these adducts via in situ-generated formaldehyde is likely a key process in the cytotoxic mechanism of DOX, DAU, and EPI. As such, the formaldehyde conjugates DOXF, DAUF,

and EPIF essentially provide the active metabolites of DOX, DAU, and EPI.

The slower nuclear release of fluorophore from cells treated with DOXF with respect to cells treated with DOX is also apparent from microscopy experiments with both sensitive and resistant tumor cells as shown in Figure 5. Note that in the early stage of fluorophore release, fluorescence is observed in the cytoplasm of sensitive cells (Figure 5, column A, row a) but less in the cytoplasm of resistant cells (Figure 5, column B, row a). This no doubt reflects the increased levels of Pgp in resistant cells (*21*), which pumps cytoplasmic drug out of cells as it is released from the DNA. Similar results were seen with DAUF and EPIF with respect to DAU and EPI (data not shown). Drug–DNA adducts formed from reaction of DOXF, DAUF, and EPIF with extracellular DNA are unstable with respect to hydrolysis back to parent drug, formaldehyde, and intact DNA (*6–9*, *11*, *22*, *25*). Consequently, the drug observed in the cytoplasm of MCF-7 cells following DOXF treatment (Figure 5, column A, row a) is probably DOX. Formation of relatively long-lived but ultimately unstable DNA lesions may afford DOXF, DAUF, and EPIF tumor cell selectivity. Cancer cells may be especially susceptible to such adducts because the cells are constantly replicating and cannot arrest their growth to allow for DNA repair. Normal cells, however, can slow their growth such that DNA repair can take place. Repair might occur by an active mechanism or simply by a passive mechanism, as observed in experiments with extracellular DNA (*6–8*, *25*).

As mentioned earlier in the Discussion, the occurrence of drug–DNA alkylation is further supported by the cell experiments with [^{3}H]DOXF. The tritium label on [^{3}H]-DOXF was present on the methylene units of DOXF, as indicated in Scheme 1. Thus, the tritium label should be bound to the cellular DNA upon formation of the drug–DNA adducts (Scheme 1). As the data in Table 1 indicate, more radioactivity was present in the DNA of [^{3}H]DOXF-treated cells relative to the controls. Furthermore, elevated ^{3}H levels were observed in DNA of MCF-7/ADR cells with respect to MCF-7 cells. This somewhat contradicts the flow cytometry data, which indicate that more DOXF is taken up by sensitive cells at a concentration of 0.5 μmol equiv/L. The reason for this discrepancy is unclear. The flow cytometer measures only the drug fluorophore, while the scintillation counter measures tritiated formaldehyde from DOXF. Perhaps the difference lies in the proportion of drug–DNA binding sites occupied by intercalated drug versus intercalated and covalently bound drug. Because of relatively rapid DOXF hydrolysis to DOX (*22*) (Scheme 1), both DOX and DOXF are available for binding to DNA. If DOX occupies the binding site, the active metabolite from partial hydrolysis of DOXF cannot. Because of the activity of the drug efflux pump, Pgp, DOX will occupy less of the binding sites in MCF-7/ADR cells. Hence, a higher proportion of intercalated and covalently bound drug may be present in MCF-7/ADR cells.

DOXF, DAUF, and EPIF Overcome Some Resistance Mechanisms. The flow cytometry measurements indicate that resistant cells take up less of the parent drugs than the sensitive cells but take up significant amounts of the respective formaldehyde conjugates. This is consistent with resistance resulting in part from overexpression of Pgp (*18*). The drug–formaldehyde conjugates may overcome efflux by Pgp because they rapidly bind DNA (*11*, *22*). Once covalently bound to DNA, the anthracycline–formaldehyde conjugates are no longer substrates for Pgp efflux. Conjugation with formaldehyde also dramatically lowers the pK_a of the protonated amine functional group (*33*) such that the nitrogens of the conjugates are unprotonated at physiological pH. This makes the conjugates poorer substrates for Pgp, since anthracycline efflux by Pgp correlates with the presence of positive charge (*34*).

Overexpression of Pgp is an important resistance mechanism in MCF-7/ADR cells (*18*, *21*). Indeed, the data in Figure 1 are consistent with diminished uptake of DOX, DAU, and EPI by MCF-7/ADR cells as a resistance mechanism for these cells. Another mechanism of resistance observed in MCF-7/ADR cells involves changes in activity of redox enzymes involved in oxidative stress and, presumably, formaldehyde production. Xenografts of MCF-7/ADR cells in nude mice have higher activity levels of superoxide dismutase and glutathione peroxidase (*32*). Both enzymes neutralize oxidative stress. Further, the level of cytochrome P450 reductase activity was lower in the xenografts. Cytochrome P450 reductase induces oxidative stress by reducing the anthracyclines in the presence of molecular oxygen to initiate redox cycling. The formaldehyde conjugates are effective against this resistance mechanism as well because they do not require drug induction of oxidative stress for production of formaldehyde.

In conclusion, we have shown that the anthracycline–formaldehyde conjugates, Daunoform, Doxoform, and Epidoxoform, target the nuclei of both sensitive and resistant MCF-7 tumor cells with formation of unstable drug–DNA adducts. Important factors in the toxicity to tumor cells are likely the quantity of drug taken up and the longevity of drug association with nuclear DNA. Although a longer residence time with DNA increases toxicity to tumor cells, an overly long residence time with irreversible DNA damage may result in the loss of selectivity for tumor cells. Possibly one or more of these anthracycline–formaldehyde conjugates has the correct reactivity with DNA for specific toxicity to anthracycline-sensitive and -resistant tumor cells with little or no toxicity to normal cells. Animal experiments are currently in progress to address this question.

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